

Generation and Evaluation of Prophylactic mRNA Vaccines Against Allergy

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Abstract

Due to the worldwide increase in allergies and a limited efficacy of therapeutic interventions, the need for prophylactic vaccination against allergies has been recognized. mRNA and DNA vaccines have demonstrated their high potential for preventing allergic sensitization by inducing an immunological bias that prevents TH2 sensitization. However, only mRNA vaccines fulfill the stringent safety requirements for vaccination of healthy children. In this chapter, we describe the generation of conventional as well as self-replicating mRNA vaccines and methods to test their prophylactic efficacy in animal models.

Key words mRNA vaccine, Self-replicating mRNA, Genetic immunization, Prophylactic, TH1/TH2 responses, Allergy, BALB/c

1 Introduction

Although therapeutic DNA vaccines against cedar and peanut allergy have recently entered clinical trials [1], it is unlikely that this type of vaccines will fulfill the stringent safety demands necessary for prophylactic vaccination in healthy individuals. mRNA vaccines have reemerged as a promising alternative that avoid risk factors associated with DNA vaccines. We have previously demonstrated that mRNA vaccines have the potential to prevent from allergic sensitization, i.e., the generation of TH2 biased immune responses characterized by high levels of allergen specific IgE and the hallmark cytokines IL-4, IL-5, and IL-13 [2, 3]. Notably, the requirements for prophylactic vaccination against allergies markedly differ from traditional vaccines. While vaccines against infectious diseases aim at inducing high titers of protective antibodies and/or cellular immunity, a prophylactic vaccine against allergy introduces an immune bias, which is subsequently boosted upon natural contact with the respective allergen. It has been shown, that even a barely detectable primary immune response induced by the vaccine, is sufficient to prevent from allergic sensitization [4]. Moreover, we

could show that memory responses after vaccination are long lasting and are maintained by repeated allergen challenges via the airways, without inducing detrimental side effects [5]. Thus, mRNA vaccines against allergic diseases meet all requirements for safe and effective prophylactic vaccines against allergies.

In this book chapter we describe the generation of conventional as well as self-replicating mRNAs from commercially available vectors. While conventional mRNA vaccines are limited to the minimal elements required for efficient translation of an antigen, self-replicating mRNA vaccines make use of a replicase, which can amplify the mRNA vaccine and trigger additional immunostimulatory pathways. A more detailed description of this vaccine type can be found in another book chapter of this series [6]. Additionally, we provide methods for quality control of the vaccines and suggestions which parameters can be optimized. Finally, we address in vivo vaccine delivery and describe methods for assessment of vaccine efficacy. Further methods to assess cellular immune responses and lung function in sensitized mice have been described in detail elsewhere [7].

2 Materials

2.1 Construction of Plasmids

1. pTNT (Promega).
2. pSinRep5 (Thermo Fisher).
3. Restriction enzymes, calf intestine alkaline phosphatase (CIAP), T4 DNA Ligase, corresponding buffers.
4. Agarose gel DNA electrophoresis equipment.
5. Gel extraction kit.

2.2 Plasmid Purification and Linearization

1. Escherichia coli XL1-blue competent cells (Thermo Fisher).
2. Standard Luria broth (LB) Medium.
3. Ampicillin.
4. Plasmid DNA preparation kit.
5. BamHI and NotI restriction enzymes and appropriate buffers.
6. MaXtract™ High Density, 2 mL (Qiagen).
7. Phenol–chloroform–isoamyl alcohol (PCI) 25:24:1.
8. Chloroform–isoamyl alcohol (CI) 24:1.
9. 3 M sodium acetate, pH 5.2, RNase free.
10. H₂O, RNase free.
11. 100 % ethanol, RNase free.
12. 70 % ethanol, RNase free.
13. Filter tips, RNase free.

2.3 *In Vitro*

Transcription of RNA

All reagents need to be RNase free.

1. 5× T7 reaction buffer: 400 mM HEPES-KOH pH 7.5, 120 mM MgCl₂, 10 mM spermidine, 200 mM DTT.
2. 5× SP6 reaction buffer: 400 mM HEPES-KOH pH 7.5, 160 mM MgCl₂, 10 mM spermidine, 200 mM DTT.
3. rNTP mix: 25 mM rATP, 25 mM rCTP, 25 mM rGTP, 25 mM rTTP.
4. T7 and SP6 RNA polymerase, high concentration (200 U/μL) (Thermo Fisher).
5. Inorganic pyrophosphatase (NEB).
6. RNase-free DNase (Thermo Fisher).
7. 5 M ammonium acetate, pH 6.2–7.5.
8. H₂O, RNase free.
9. 70 % ethanol, RNase free.
10. Filter tips, RNase free.

2.4 **RNA Capping**

2.4.1 All Reagents Need to Be RNase Free

1. ScriptCap m⁷G Capping Kit (CELLSCRIPT).
2. 5 M ammonium acetate, pH 6.2–7.5.
3. H₂O, RNase free.
4. 70 % ethanol, RNase free.
5. Filter tips, RNase free.

2.5 **RNA Quality Control**

2.5.1 All Reagents Need to Be RNase Free

1. Agarose.
2. 20× RNA buffer: 500 mM BES or MOPS, 300 mM sodium acetate, 10 mM Na₂EDTA, pH 6.8.
3. RNA loading buffer (FOFAL): To make 1 mL add 50 μL 20× RNA buffer, 500 μL formamide (>99%), 184 μL formaldehyde (37%), 134 μL glycerol (87%), 5 μL ethidium bromide (10 mg/mL), 117 μL H₂O, RNase free.
4. RNA-marker for agarose gel electrophoresis.
5. BHK-21 cell line (ATCC number CCL-10).
6. BHK-21 growth medium: DMEM, 10% FBS, 4.5 g/L glucose, 4 mM L-glutamine, 2.2 g/L NaHCO₃.
7. TurboFect transfection reagent (Thermo Fisher).
8. DPBS without Ca⁺⁺ and Mg⁺⁺.
9. Lysis solution: 100 mM potassium phosphate, pH 7.8, 0.2% Triton X-100.
10. Trichloroacetic acid (TCA), 30 % w/v.
11. 2× SDS-sample-buffer: 200 mM β-mercaptoethanol, 4 % (w/v) SDS, 20 % (v/v) glycerol, 0.2 % (w/v) bromophenol blue, 100 mM Tris-HCl, pH 6.8.

12. Equipment for SDS-PAGE and western blotting.
13. Immune serum against the protein encoded by the RNA (*see Note 1*).
14. HRP-labeled anti-mouse-IgG1 secondary antibody.

2.6 Immunization and Allergic Sensitization

1. BALB/c mice (female, 6–10 weeks of age).
2. 27-gauge needles.
3. 1-mL syringes.
4. Alu-Gel-S (Serva).
5. Purified allergen-encoding mRNA.
6. 5× Ringer's Solution with optimized CaCl_2 concentration: 513.5 mM NaCl, 26.5 mM KCl, 3.75 mM CaCl_2 (*see Note 2*).
7. Recombinant allergen.
8. 10× endotoxin-free phosphate buffered saline (PBS), pH 7.5 (Sigma).
9. Endotoxin-free H_2O .
10. Curved forceps.
11. Isoflurane anesthesia machine suitable for rodents.

2.7 IgG1 and IgG2a Subclass ELISA

1. White 96-well high-bind flat bottom ELISA plates (Greiner).
2. Standard PBS, pH 7.5.
3. Recombinant allergen.
4. Wash buffer: PBS, 0.1 % (v/v) Tween-20.
5. Blocking buffer: PBS, 0.1 % (v/v) Tween-20, 2 % (w/v) blotting-grade skim milk.
6. Detection antibodies: anti-mouse IgG1 and IgG2a, HRP-conjugated.
7. BM Chemiluminescence ELISA substrate (POD) (Roche).
8. Luminescence microplate reader.

2.8 Basophil Release Assay

1. Rat basophil leukemia (RBL)-2H3 cell line (ATCC no. CRL-2256; DSMZ no. ACC 312).
2. Culture medium: 70 % MEM (with Earle's salts) and 20 % RPMI 1640 supplemented with 10 % FBS, 2 mM L-glutamine, 1 % penicillin/streptomycin, 2 mM sodium pyruvate.
3. Cell culture flasks with 25 cm^2 , 75 cm^2 , and 175 cm^2 area.
4. DPBS (Sigma).
5. Trypsin–ethylene-diamine tetraacetic acid (EDTA): 0.05 % trypsin, 0.02 % EDTA in DPBS (Sigma).
6. 96-well flat-bottom cell culture plates.

7. Tyrode's buffer: 9.6 g Tyrode's salts (Sigma), 1 g NaHCO_3/L ; adjust pH to 7.2 with NaOH or HCl; add 0.1 % BSA freshly before use.
8. Citrate buffer: 0.1 M citric acid or sodium citrate in H_2O ; adjust pH to 4.5 with NaOH.
9. Glycine buffer: 15 g glycine, 11.7 g NaCl/L ; adjust pH to 10.7 with NaOH.
10. 4-MUG (4-methyl umbelliferyl-N-acetyl- β -D-glucosaminide) (Sigma): 10 mM solution in DMSO; store aliquots of 80 μL or multiples thereof at -70°C .
11. 10 % Triton X-100.
12. Non-sterile 96-well flat-bottom microtiter plates.
13. Fluorescence microplate reader.

3 Methods

3.1 Construction of Plasmids for RNA Transcription

Plasmid vectors for transcription of mRNA vaccines employ a promoter specific for a viral RNA polymerase (typically T7 or SP6), which drives downstream transcription of the mRNA of interest. Elements to stabilize the transcribed RNA are usually incorporated into the vector. These include untranslated sequences before (5' UTR), and/or after (3' UTR) the cDNA sequence encoding the gene of interest [8]. The 3' UTR also contains a synthetic poly(A) tail, which is important for efficient translation of the protein. Longer poly(A) tails usually result in increased mRNA half-life and more efficient protein translation [9]. A commercially available vector employing these features is pTNT (Promega). This vector contains tandem SP6 and T7 promoters, a 5' UTR from rabbit β -globin [10], and a synthetic poly(A)₃₀ tail (*see* Fig. 1).

Plasmid vectors for transcription of self-replicating mRNA have to fulfill different requirements. These vectors are used for transcription of a so called genomic RNA, which encodes the non-structural proteins 1–4 of an alphavirus (e.g., Sindbis or Semliki Forest Virus), which form the viral replicase. Downstream of the replicase lies a subgenomic promoter, also called 24-nucleotide (nt) conserved sequence element (CSE) [11], which drives the expression of the gene of interest. This is followed by a viral 3' UTR including a conserved 19-nt CSE, which forms the core promoter for synthesis of negative strand RNA [12], and a synthetic poly(A) tail. Interaction of the viral 3' and 5' UTRs is necessary to initiate replication and to regulate both minus- and plus-strand synthesis [13].

The pSFV and pSinRep5 (Fig. 1) vectors are available from Thermo Scientific and can be used to generate self-replicating RNAs employing replicases from SFV and Sindbis virus, respectively.

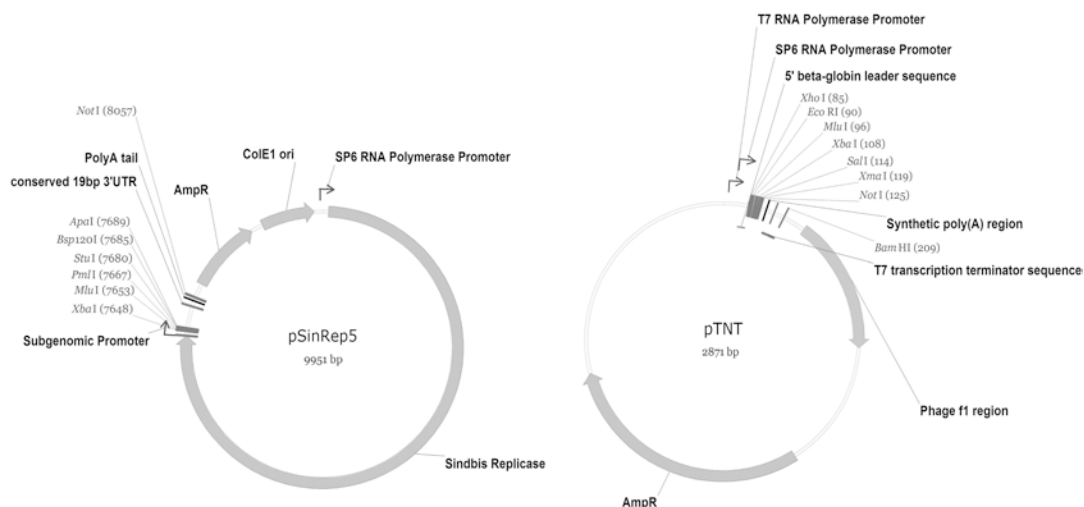


Fig. 1 Commercially available vectors for in vitro transcription of self-replicating (pSinRep5) or conventional (pTNT) mRNA vaccines

Constructions of pTNT-P5 and pSin-P5 vectors can be performed by standard recombinant DNA methods. Vectors pTNT and pSinRep5 are linearized with XbaI and treated with calf intestine alkaline phosphatase (CIAP) to prevent religation. The cDNA encoding Phl p 5 is excised from pCMV-Phl p 5 [14] using NheI/XbaI and ligated into the linearized vectors. The resulting vectors are named pTNT-P5 and pSin-P5, respectively.

3.2 Plasmid Purification and Linearization

Plasmid templates can be prepared with any commercially available plasmid preparation kit that gives endotoxin-free plasmid DNA of good quality. This plasmid DNA has to be linearized after the poly(A) tail before mRNA can be transcribed (*see Note 3*).

Complete linearization of plasmid DNA is important to avoid circular transcription, resulting in RNA of incorrect size. It is preferable to generate a larger batch of linearized plasmid DNA which can then be stored at -20°C .

1. Add 200 μg of plasmid DNA (pTNT-P5 or pSin-P5) dissolved in H_2O to a 1.5 mL reaction tube.
2. Add 50 μL of appropriate 10 $\times$ reaction buffer.
3. Add 20 μL of BamHI (10 U/ μL), or NotI (10 U/ μL) for linearization of pTNT-P5 or pSin-P5, respectively (*see Note 4*).
4. Fill up to 500 μL with H_2O , vortex, and incubate for 2 h at 37°C (*see Note 5*).
5. From this point on work under RNase-free conditions: wear gloves, use certified RNase-free filter tips, reaction tubes, and reagents.

6. Prepare 2 MaXtract™ High Density columns by centrifugation at $1500 \times g$ for 2–3 min.
7. Transfer reaction to a MaXtract™ High Density column (*see Note 6*).
8. Add 500 μ L PCI (25:24:1) and mix thoroughly by repeated inversion.
9. Centrifuge at $1500 \times g$ for 5 min to separate phases.
10. Transfer aqueous phase to the second MaXtract™ High Density column prepared at **step 6**.
11. Add 500 μ L CI (24:1) and mix thoroughly by repeated inversion.
12. Centrifuge at $1500 \times g$ for 5 min to separate phases.
13. Transfer the aqueous phase to a new reaction tube.
14. Add 1/10 Vol of 3 M sodium acetate, pH 5.2 and vortex.
15. Add 2 Vol of 100% ethanol and vortex.
16. Centrifuge at $16,000 \times g$ for 10 min at RT.
17. Wash pellet 3 times with 70% ethanol.
18. Air-dry pellet and dissolve in 100 μ L H₂O.
19. Measure concentration and check complete linearization on 1% agarose gel.
20. Store linearized plasmid DNA at -20°C .

3.3 *In Vitro* Transcription of RNA

RNA is in vitro-transcribed from the linearized plasmid DNA using T7 or SP6 RNA polymerase. Milligram quantities of RNA can be produced by using optimized buffers and high concentrations of rNTPs [15] and inorganic pyrophosphatase [16], which prevents the inhibitory effects of pyrophosphate released during ribonucleoside triphosphate incorporation. We have successfully employed homemade buffers as described in this chapter; however, all necessary components are available in kits from Promega (RiboMAX™ Large Scale RNA Production Systems).

Conventional mRNA encoding Phl p 5 can be transcribed from linearized pTNT-P5 template DNA using T7 or SP6 polymerase. A 1 mL reaction results in approximately 2–5 mg of RNA. Similarly, self-replicating RNA can be transcribed from linearized pSin-P5 using SP6 polymerase. A 1 mL reaction will yield approximately 1 mg of self-replicating RNA.

1. Add 200 μ L of 5 $\times$ T7 or SP6 reaction buffer to a 1.5 mL reaction tube.
2. Add 200 μ L of rNTP mix.
3. Add 50–100 μ g of linearized pTNT-P5 or pSin-P5.
4. Add 30 U T7 or SP6 RNA polymerase per μ g template DNA.

5. Add 15 U inorganic pyrophosphatase.
6. Fill up to 1 mL with H₂O.
7. Mix by repeated inversion.
8. Incubate at 37 °C for 2–3 h.
9. Add RNase-Free DNase to a concentration of 1 U/μg of template DNA.
10. Mix by repeated inversion.
11. Incubate at 37 °C for 15 min.
12. Add 1 mL of 5 M ammonium acetate, pH 6.2–7.5.
13. Mix by repeated inversion.
14. Incubate on ice for 15 min.
15. Centrifuge at 16,000 × *g* for 15 min at 4 °C.
16. Wash pellet in 70% EtOH.
17. Air-dry pellet and dissolve in 100 μL H₂O (*see Note 7*).
18. Measure RNA concentration (*see Note 8*).

3.4 RNA Capping

A 7-methyl-guanosine cap structure is essential for mRNA stability and efficient translation [17]. Cap analogs can be incorporated during the transcription process. Alternatively, cap 0 structures (m⁷G(5')ppp(5')NpN) can be added to the 5' end of RNA using vaccinia virus capping enzyme [18]. The latter method has the advantage that up to 100% of transcripts can be capped. In this chapter, we describe the enzymatic capping approach using the ScriptCap m⁷G Capping Kit (CELLSCRIPT). The kit contains the vaccinia virus capping enzyme including all three enzymatic activities needed for cap construction, i.e., mRNA triphosphatase, guanylyltransferase, and guanine-7-methyltransferase.

1. Add 50–60 μg in vitro-transcribed RNA to a 1.5 mL reaction tube and fill up to 68.5 μl with RNase-free water.
2. Heat-denature sample at 65 °C for 5–10 min and then immediately transfer on ice.
3. On ice add in the following order and mix after each step:
 - (a) 10 μL 10× ScripCap buffer.
 - (b) 10 μL rGTP (10 mM).
 - (c) 5 μL S-adenosyl methionine (SAM) (2 mM).
 - (d) 4 μL ScriptCap Capping Enzyme (10 U/μl) (*see Note 9*).
4. Fill up to 100 μL with RNase-free water and mix.
5. Incubate for 30–60 min at 37 °C.
6. Add 100 μL of 5 M ammonium acetate, pH 6.2–7.5.
7. Mix by repeated inversion.

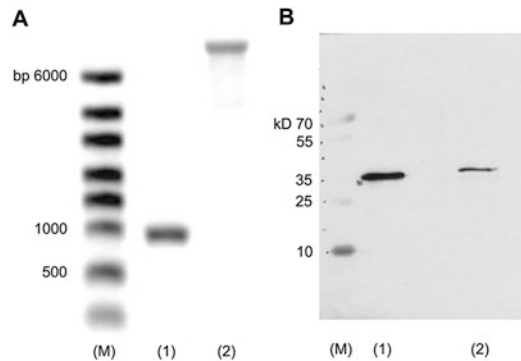


Fig. 2 RNA vaccine quality control. **(a)** Correct length of transcribed RNA is assessed by denaturing agarose gel electrophoresis. (M) marker, (1) Phl p 5 mRNA transcribed from pTNT-P5, (2) self-replicating RNA transcribed from pSin-Rep5-P5. **(b)** Western blot of cell lysates of BHK-21 cells transfected with conventional (1) or self-replicating (2) RNA encoding Phl p 5

8. Incubate on ice for 15 min.
9. Centrifuge at $16,000 \times g$ for 15 min at 4 °C.
10. Wash pellet in 70 % EtOH.
11. Air-dry pellet and dissolve in 100 μ L H₂O.
12. Measure RNA concentration.

3.5 RNA Quality Control

3.5.1 Denaturing Agarose Gel Electrophoresis

Quality control of capped mRNA is done by means of denaturing agarose gel electrophoresis, in vitro transfection, and western blot analysis.

Integrity and correct length of the transcribed RNA is checked by denaturing agarose gel electrophoresis. Ideally, transcripts show as a single band of the expected size (Fig. 2). Longer transcripts point to insufficient linearization of the plasmid template or the presence of cryptic antisense promoters. Smaller transcripts can be the result of RNA secondary structures such as poly-T regions or repeats (*see Note 10*).

For 20 mL of a 1 % agarose gel:

1. Add 0.2 g agarose to 17.6 mL RNase-free water.
2. Boil up in microwave until agarose is completely dissolved.
3. Cool down to approximately 60 °C.
4. Under a fume hood add 1 mL of 20 $\times$ RNA buffer and 1.4 mL of 37 % formaldehyde solution. Mix by swirling.
5. Cast gel.
6. Mix 1 μ g of RNA with 1/10 Vol of RNA loading buffer (FOFAL).

7. Add an appropriate amount of RNA marker (ready to use) to a reaction tube.
8. Incubate RNA and RNA marker at 95 °C for 5 min and put on ice afterwards.
9. Immerse gel in 1× RNA buffer, add RNA and marker and run at 100 V.

3.5.2 *In Vitro* Transfection and Western Blot

Translation efficacy of the RNA can be tested by in vitro transfection of BHK-21 cells. Effective translation is dependent on the integrity and secondary structure of the RNA, and presence of a cap 0 structure. Also codon usage may affect translation efficacy (*see* **Note 11**).

1. On the day before transfection, seed BHK-21 cells into 24-well tissue culture plate at a density of 50–70% (2.5×10^5 cells per well).
2. For each transfection dilute 1 µg of RNA in 100 µL DMEM without serum and vortex.
3. Add 1 µL of TurboFect.
4. Mix solution by pipetting gently up and down.
5. Incubate for 15–20 min at RT.
6. Add 100 µL of the polymer/RNA mixture dropwise to the well.
7. Incubate for 24 h at 37 °C, 5 % CO₂.
8. The next day, remove supernatant and wash wells once with 500 µL DPBS.
9. Add 100 µL of lysis solution.
10. Incubate 1–2 min at 37 °C.
11. Resuspend cells by pipetting up and down and thoroughly rinse the well bottom.
12. Transfer lysate to a fresh tube.
13. Precipitate proteins by adding 100 µL of 30 % TCA.
14. Incubate on ice for 10 min.
15. Centrifuge at $16,000 \times g$ for 10 min at 4 °C.
16. Discard supernatant.
17. Resuspend pellet in 50 µL SDS-sample-buffer (buffer turns yellow).
18. Add 1 M NaOH until sample becomes blue again.
19. Incubate at 95 °C for 10 min.
20. Add 15 µL of sample to a 12.75 % polyacrylamide gel.
21. Run SDS-PAGE.
22. Perform western blot and stain protein with suitable antibody (*see* **Note 12**).

3.6 Immunization and Allergic Sensitization of Mice

Due to the fact that BALB/c mice are prone to develop TH2-biased immune responses, they are frequently employed for allergy models. Following sensitization with recombinant allergen together with adjuvants such as aluminum hydroxide (Alu-Gel-S), high titers of allergen-specific IgE and elevated levels of IL-4, IL-5, and IL-13 can be detected in these animals. Furthermore, the ratio of allergen-specific IgG1:IgG2a subclass antibodies is elevated in sensitized mice, indicative of a TH2-dominated response type. By immunizing mice with allergen-encoding mRNA vaccines prior to sensitization, the formation of IgE and IgG1 as well as the production of allergy-associated cytokines can be prevented.

A typical experimental setup for protective mRNA immunization against allergy requires 2–3 immunizations at weekly intervals followed by 2–3 sensitization rounds. To avoid antigen-independent suppression of TH2 sensitization, an interval of at least 2 weeks between vaccination and sensitization should be maintained. Longer intervals are possible, as vaccine memory lasts for at least 9 months [5]. For control purposes, groups of animals receiving mRNA encoding an irrelevant antigen/allergen and/or only sensitizations have to be included.

3.6.1 Intradermal mRNA Immunization

1. Prepare allergen-encoding mRNA in a volume of 200 μ L 1 $\times$ Ringer's solution per mouse and keep the RNA on ice until injected (*see* **Note 13**).
2. Carefully shave the back of the mice with an electric clipper.
3. Anesthetize the mice by using an isoflurane anesthesia machine and keep them under anesthesia during the whole procedure.
4. Lift a small fold of the skin with the forceps.
5. Inject a small amount of the mRNA superficially into this fold (*see* **Note 14**).
6. Repeat this step 5 times to distribute the solution into several injection sites.

3.6.2 Subcutaneous Sensitization

1. Prepare a mixture of 1–5 μ g recombinant allergen (*see* **Note 15**) with 100 μ L Alu-Gel-S and 20 μ L endotoxin-free 10 $\times$ PBS and fill up with endotoxin-free water to a volume of 200 μ L per mouse.
2. Let protein-adjuvant complex formation take place by shaking the solution in a large enough tube at room temperature for at least 2 h.
3. Distribute the solution between two sites on the shaved back of the mice by lifting up the skin and injecting subcutaneously.
4. Repeat the sensitization after 2 weeks (*see* **Note 16**).

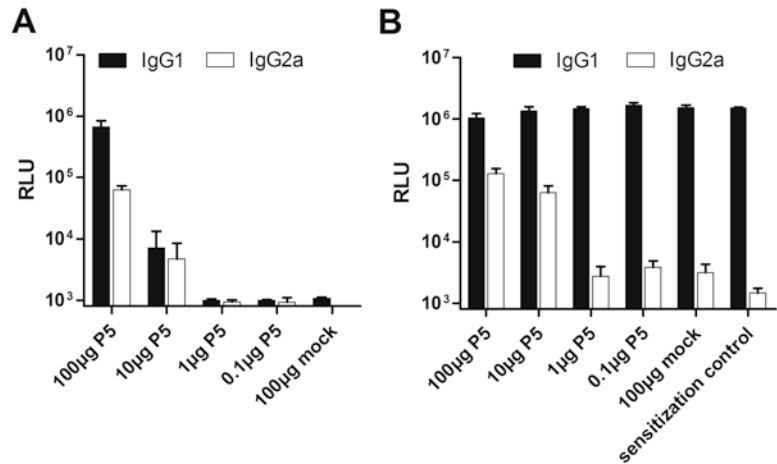


Fig. 3 (a) Phl p 5-specific IgG1 and IgG2a responses after vaccination of mice ($n=5$) with 0.1–100 µg of conventional mRNA encoding Phl p 5, or 100 µg of mRNA encoding an irrelevant antigen (mock). (b) IgG1 and IgG2a level after sensitization with recombinant Phl p 5/Alu-gel-S. A boost of IgG2a in the 100 µg and 10 µg groups indicates successful induction of TH1 memory and correlates with protection from sensitization. All sera were diluted 1:1000. Data are shown as means $\pm$ SEM

3.7 Measurement of Antibody Subclasses

To test whether prophylactic immunization has worked out, the easiest parameter to measure is the ratio of IgG1:IgG2a in sera. Although the humoral responses after immunization might be barely detectable, a boost of IgG2a titers after allergic sensitization is a strong indicator of a TH1 bias set by the RNA vaccine and successful protection (Fig. 3).

1. Coat ELISA plates with 50 µL/well of a 1 µg/mL allergen solution in PBS at 4 °C overnight.
2. Wash wells 3 times with 200 µL PBS, 0.1 % Tween-20.
3. Block with 200 µL blocking buffer/well for 1 h at RT.
4. Wash wells 3 times with 200 µL PBS, 0.1 % Tween-20.
5. Add 50 µL of serial dilutions of individual sera in blocking buffer to the wells and incubate for 1 h at room temperature.
6. Wash wells 5 times with 200 µL PBS, 0.1 % Tween-20. Incubate 1 min between each wash.
7. Add 50 µL of detection antibodies diluted in blocking buffer to the wells and incubate for 1 h at room temperature.
8. Wash wells 5 times with 200 µL PBS, 0.1 % Tween-20. Incubate 1 min between each wash.
9. Prepare Chemiluminescence substrate according to the manufacturer's instructions and dilute the substrate 1:2 with H₂O.

10. Add 50 μL of the substrate to the wells and incubate for 3 min at room temperature.
11. Measure chemiluminescence in photon counts per second in a luminometer.

3.8 Basophil Release Assay

To assess the amount of free allergen-specific IgE in sera of sensitized mice, basophils expressing Fc ϵ RI, the high affinity receptor for IgE, are passively loaded with IgE. Addition of the respective allergen leads to cross-linking of IgE and subsequently to mediator release. One of these mediators, β -hexosaminidase, cleaves the substrate 4-MUG, leading to formation of cleavage products detectable by fluorescence spectroscopy.

In contrast to measurement of IgE by ELISA, the RBL release assay provides a functional readout for IgE-mediated degranulation. Additionally, the amount of cell-bound IgE in the blood of sensitized animals can be determined by a basophil activation test, which has been described in detail elsewhere [7].

3.8.1 Culture of RBL Cells

The RBL-2H3 cells are adherent, fibroblast-like cells forming monolayers. They are cultured in a mixture of MEM and RPMI (*see Note 17*) with supplements in an incubator at 37 °C, 95% RH, 5% CO₂.

1. Culture cells in 25 cm², 75 cm², or 175 cm² cell culture flasks with 5 mL, 20 mL, or 50 mL culture medium, respectively.
2. Split culture before becoming confluent (*see Note 18*).
3. Remove culture medium.
4. Wash cells 3 times with DPBS (*see Note 19*).
5. Cover the cell monolayer with pre-warmed trypsin–EDTA.
6. Place culture flask in the incubator until cells detach (*see Note 20*).
7. Add warm culture medium (*see Note 21*).
8. Rinse off the cells (*see Note 22*) and transfer them into a fresh tube.
9. Centrifuge for 10 min at 300 $\times g$.
10. Discard the supernatant and dissolve the cell pellet in culture medium.
11. Transfer cells into fresh culture flasks at the desired dilution.
12. For storage, freeze cells in 70% medium, 20% FBS, 10% DMSO on liquid nitrogen vapor or at –70 °C.

3.8.2 Mediator Release

1. Harvest cells from a dense culture as described under Subheading 3.8.1 (*see Note 23*).
2. Count cells and plate them in culture medium at $7 \times 10^5/\text{mL}$ in 100 μL per well of a 96-well flat-bottom tissue culture plate.

3. Place the plate in the incubator overnight.
4. Add serial dilutions of sera (*see* **Note 24**) and incubate for 2 h in the incubator. Wells for background and maximum release are left untreated at this point.
5. Discard the supernatant and dry the plate by tapping on a paper towel.
6. Carefully wash the cells 3 times by adding 200 μL Tyrode's buffer.
7. Add 100 μL Tyrode's buffer containing dilutions of allergen or allergen extract (*see* **Note 25**). For maximum release, 10 μL of a 10% Triton solution is added to the maximum release wells. Background wells receive Tyrode's buffer only.
8. Place the plate in the incubator for 1 h.
9. Carefully resuspend the solution contained in the maximum release wells.
10. Transfer 50 μL supernatant of each well into a fresh, non-sterile 96-well flat-bottom plate.
11. Thaw 4-MUG and add 80 μL thereof per 5 mL of citrate buffer to prepare assay solution.
12. Add 50 μL assay solution to each supernatant and incubate for 1 h at 37 °C.
13. Add 100 μL glycine buffer per well.
14. Measure fluorescence (in relative fluorescence units, rfu) at λ_{ex} 360 nm, λ_{em} 465 nm in a fluorescence microplate reader.
15. Calculate specific release in percent according to the following equation:
$$\frac{((\text{experimental}_{\text{rfu}} - \text{background}_{\text{rfu}}))}{(\text{maximum}_{\text{rfu}} - \text{background}_{\text{rfu}})} \times 100.$$

4 Notes

1. You can use immune sera from previous experiments of mice sensitized with the recombinant protein adjuvanted with Alu-Gel-S as described in Subheading 3.6.2.
2. The *in vivo* uptake of naked mRNA strongly depends on the presence of Ca^{++} ions [19]. We optimized the CaCl_2 concentration in Ringer's solution using *in vivo* expression of luciferase mRNA as readout.
3. Although pTNT contains a T7 terminator after the poly(A) tail, this termination is not 100% efficient. Therefore, linearization of the vector is necessary to avoid too long transcripts.
4. If possible, restriction enzymes producing 5' overhangs should be used as 3' overhangs may initiate transcription and generated unwanted transcripts.

5. Although the digest should be finished in less time, we found that longer incubation gives better reproducible results.
6. We found that purification of linearized template DNA by organic extraction results in the best transcription rates. It is important to avoid any phenol contamination of the template DNA as this would inhibit downstream enzymatic reactions. Therefore, use of MaXtract gel, which separates the aqueous and organic phase, is highly recommended.
7. Do not overdry the pellet. Depending on the pellet size, solubilization of the RNA can take some time. We usually incubate the pellet in water over night at 4 °C and resuspend on the next day. If the pellet is still not completely dissolved, incubate at 50 °C and vortex every 2–3 min until fully dissolved.
8. Measurement of RNA concentration by UV absorption at 280 nm can lead to an overestimation due to the presence of free rNTPs. Although ammonium acetate precipitation minimizes contamination with free rNTPs we routinely experience an overestimation of ~10% by UV absorption measurement. To determine the exact RNA concentration, the use of RNA intercalating dyes, which are not affected by the presence of free rNTPs are recommended, e.g., using the Quant-iT RNA Assay Kit (Thermo Fisher).
9. To save enzyme, we tested the minimum amount of Capping Enzyme required to achieve 100% incorporation of radioactively labeled SAM. We found that by increasing the incubation time to 2–2.5 h, 0.8 µL of capping enzyme are sufficient to incorporate the same amount of methyl groups as 4 µL.
10. Though in vitro-transcribed RNA is often of high enough purity for animal experiments, large transcripts or GMP requirements can make additional chromatographic purification steps necessary. A protocol to further purify in vitro-transcribed RNA by size exclusion chromatography can be found here [20].
11. RNA vaccines that fail to translate after in vitro transfection may have to be optimized. Several companies offer services for optimizing codon usage and secondary structural elements of the RNA encoding the antigen of interest.
12. We use sera from mice immunized with recombinant Phl p 5 adjuvanted with Alu-Gel-S at a dilution of 1:5000. Detection is performed with a HRP-conjugated goat anti-mouse IgG1 secondary antibody. Use a dilution as recommended by the manufacturer.
13. 10–100 µg of conventional mRNA are required for protective vaccination, depending on the antigen. For self-replicating RNA, five to tenfold lower doses are sufficient [21].
14. Intradermal injection leads to formation of a small blister, which persists for several minutes. If the solution quickly disappears instead, the injection has been delivered into subcutaneous tissue.

15. Depending on the allergenicity of the protein used, the amount necessary for proper sensitization may vary and has to be tested on a case-by-case basis.
16. For weak allergens even a third sensitization round might be necessary. Take blood at regular intervals and check the sensitization status by mediator release assay.
17. Other media formulations are also frequently used, including RPMI 1640 alone with supplements. However, a higher proportion of RPMI may increase the proliferation rate of the cells, but potentially decreases production of mediators.
18. Usually, cells have to be split every 3–4 days at a dilution of 1:10. If cells are growing too dense, they tend to accumulate in suspension instead of adhering to the plastic. It is recommended to check the density/quality of the cells under the microscope before performing an assay.
19. Washing is essential to remove any traces of serum as this contains trypsin inhibitor.
20. This usually takes about 5 min. You can check in an inverted microscope whether the cell layer is already dispersed and if so, quickly proceed to the next step.
21. This has to be done as quickly as possible after the cells have been detached to dilute the trypsin–EDTA. Longer incubation with trypsin leads to reduced numbers of receptors on the cell surface and hence can diminish receptor cross-linking.
22. Avoid tapping against the culture flask as this may lead to clogging of the cells.
23. It has been demonstrated that histamine release capacity may be seriously reduced after too much subculturing [22]. This may also apply for release of β -hexosaminidase. Hence, we recommend starting a new culture after approx. 30 passages.
24. We recommend starting at a dilution of 1:50. If this results in weak or undetectable mediator release, the sensitization protocol might have failed.
25. A standard concentration for recombinant or purified allergens is 0.1 $\mu\text{g}/\text{mL}$. For allergen extracts we recommend to start with 1 $\mu\text{g}/\text{mL}$.

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